



Seasonal ionic and metabolic changes in Aleppo pines growing on mine tailings under Mediterranean semi-arid climate

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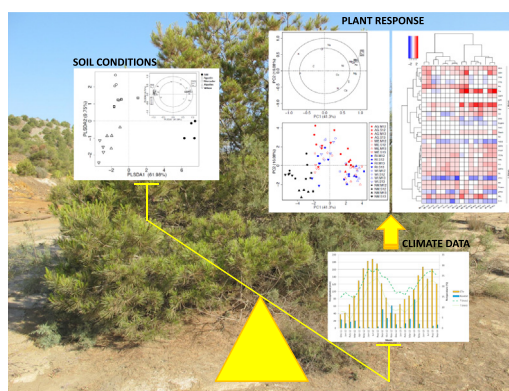
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HIGHLIGHTS

- Seasonal factors influenced needle metabolism more markedly than soil factors did.
- Higher GSH and phenol antioxidants let needles to cope with higher HMMs in spring.
- RWC was maintained in summer needles mainly by accumulation of monovalent ions.
- Lower chlorophylls and higher carotenoids increased photoprotection in summer needles.

GRAPHICAL ABSTRACT



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ABSTRACT

Aleppo pine is the most abundant conifer species in Mediterranean basin. Knowledge of adaptive mechanisms to cope with different environmental stresses simultaneously is necessary to improve its resilience to the predicted climatic changes and anthropogenic stressors, such as heavy metal/metalloid(s) (HMMs) pollution. Here, one year-old needles and rhizosphere soil samples from five mining and non-mining (NM) populations of Aleppo pines grown spontaneously in SE Spain were sampled in two consecutive years during spring and summer. Quantitative determination of a wide suite of edaphic, biochemical, and physiological parameters was performed, including soil physicochemical properties, ionome profile, foliar redox components, primary and secondary metabolites. Mining rhizosphere soils were characterized by elevated contents of HMMs, particularly lead and zinc, and low carbon, nitrogen and potassium levels. Multivariate data analysis based on needle ionome and antioxidative/oxidative parameters revealed a clear distinction between seasons irrespective of the population considered. Spring needles were characterized by higher levels of HMMs, sulfur, glutathione (GSH), proanthocyanidins (PAs), and soluble phenols (TPC), whereas reduced chlorophylls and increased levels of carotenoids, relative water content and K^+ , Na^+ and Cl^- typified summer needles. In general mining populations had higher levels of ascorbate, and TPC, and exhibited higher antioxidant activities than the NM population. This could contribute to prevent oxidative injury induced by HMMs. Taken together, results suggest that seasonal factors have a more marked effect on the metabolism of the Aleppo pine populations studied than that exerted by soil conditions. This effect could be mediated by water availability in surface soil layers. If this conclusion is right, predicted rainfall reduction and temperature increase in the Mediterranean basin associated to global climate

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change would lead to pine needle metabolism to express the summer pattern for more prolonged periods. This, in turn, could negatively affect the performance of Aleppo pine populations.

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1. Introduction

Aleppo pine (*Pinus halepensis* Mill.) is the most widely distributed conifer species in the Mediterranean region (Maseyk et al., 2008; Querejeta et al., 2008). In this area the species exhibits a bimodal growth pattern, with two optimal growth periods one in spring and a second in fall associated with favorable growing conditions (i.e., mild temperatures and adequate soil-water availability) (López-Serrano et al., 2005; Pacheco et al., 2017). Although this species is noted for its high resilience to heat and drought stress (Maseyk et al., 2008; Wellburn et al., 1996), climate warming is expected to have negative effects on tree survival and development, according to the Intergovernmental Panel on Climate Change 2014 (IPCC, 2014; <http://ipcc.ch/>). Moreover, growth rate reductions of forest trees can be aggravated as a consequence of increased concentrations of hazardous pollutants such as heavy metal and metal(loid)s (HMMs), which are particularly prevalent in areas subjected to intense mining activity (Panagos et al., 2013). The ecological consequences of the simultaneous occurrence of natural and anthropogenic stressors are difficult to predict because they cannot be inferred from individual stress studies, especially if the stress combinations result in antagonistic or conflicting responses (Choudhury et al., 2017; Suzuki et al., 2014).

Under natural conditions, plants have evolved a complex network of signal transduction pathways to cope with multiple environmental stresses occurring simultaneously. Whether a plant is able to acclimate to these challenging environmental conditions or not is going to be, ultimately, determined by the appropriate signaling and coordination of plant responses (Harfouche et al., 2014; Urano et al., 2010; You and Chan, 2015). Extensive evidence now strongly supports that reactive oxygen species (ROS) are key signal transduction molecules in plant stress signaling (Mittler, 2017), although elevated ROS levels, above a physiological threshold, can cause oxidative damage to biomolecules and cellular structures (De Gara et al., 2010). Increased production of ROS in plant cells has been widely shown under abiotic stress conditions, including HMM exposure (Gill and Tuteja, 2010; Schützendübel and Polle, 2002; You and Chan, 2015), as well as in different stress combinations (Choudhury et al., 2017; Suzuki et al., 2014). To keep ROS steady-state concentrations low, plants possess a particularly complex and redundant ROS-scavenging system, in which enzymes and metabolites are linked in a network of reactions (De Gara et al., 2010). Recent “omics” studies have highlighted that antioxidant defense machinery can play an important role not only in plant HMM-tolerance mechanisms (Dalcorso et al., 2013; Hossain and Komatsu, 2012; Singh et al., 2015) but also in the response of plants to stress combinations (Suzuki et al., 2014; Zandalinas et al., 2017). Indeed, the induction of ROS-scavenging enzymes, as well as a high content of both primary antioxidants, i.e., ascorbate (AA) and glutathione (GSH), and secondary antioxidants, such as carotenoids, proline (Choudhury et al., 2017), and different phenolic compounds (Martinez et al., 2016), were found to have a key role in plant acclimation to stress combinations. In fact, the diversity and plasticity of phenolic compounds are considered to play a key role in plant defense mechanisms towards biotic and abiotic stresses (Agati et al., 2012; Brunetti et al., 2015; Pourcel et al., 2007). Phenylalanine ammonia-lyase (PAL), the key enzyme in controlling phenolic biosynthesis, and the large family of secreted class III plant peroxidases (PRX), which catalyze the oxidation of a wide variety of phenolic compounds using hydrogen peroxide as the electron acceptor, have also been reported to be stimulated by infection and environmental stress (Almagro et al., 2009; Dixon and Paiva, 1995). In addition,

ionomics approaches have revealed that the study of shoot ionome, which represents the mineral nutrient and trace element composition of a plant (Salt et al., 2008), could potentially be used as a tool to detect specific physiological responses to environmental variation, or nutritional statuses (Baxter et al., 2008).

Metalliferous mining wastes represent very stringent conditions for plant growth because of nutrient deficiencies, high HMM content and salinity (Tordoff et al., 2000). Nevertheless, several studies have described the spontaneous colonization of HMM-enriched mine tailings by Aleppo pine in semi-arid areas (Parraga-Aguado et al., 2014, and refs. herein). Recently, woody and tree species have gained increasing interest in mine reclamation programs because of their massive and deep root systems (Luo et al., 2016). Although recent achievements in the study of the molecular responses to single stresses have been reported (Harfouche et al., 2014), the physiological and molecular mechanisms underlying the adaptation to HMMs under semi-arid Mediterranean conditions in woody plants are not clearly understood.

With this background, the overarching aim of the current work is to evaluate metabolic adjustments in response to the harsh conditions of mine tailings during both a favorable and a less-favorable growing season in Aleppo pines. To address this aim, a comparison of the antioxidative/oxidative profile, needle ionomics, physiological and edaphic parameters were carried out among five Aleppo pine populations growing either in a non-mining site (NM), or in multi-metal(loid) polluted mining tailings, located in the Cartagena-La Unión Mining District (SE Spain) during late spring (May) and late summer (September) in two consecutive years (2012 and 2013). Moreover, different dimensionality reduction and classification statistical methods were performed to identify inter-correlations among the different physiological and antioxidative/oxidative parameters evaluated, as well as possible associations between plant markers, concentrations of nutrients/metal(loid)s and soil parameters.

This work is framed within a larger study devoted to examine the oxidative stress signatures and the metabolic adjustments in response to the adverse conditions of mine-tailings under semi-arid Mediterranean conditions in different pioneer plant species, including both herbaceous (López-Orenes et al., 2018, 2017) and woody plants.

2. Materials and methods

2.1. Plant and soil sampling

Aleppo pine needles were obtained from mature trees growing spontaneously in the Cartagena-La Unión Mining District (SE of the Iberian Peninsula) in four different tailings piles known as Agustín (37°36′20″N, 0°50′15″W), Mercader (37°36′15″N, 0°50′04″W), Ripolles (37°36′18″N, 0°50′10″W), and Wikon (37°36′15″N, 0°50′08″W), and in a non mining-impacted forest (37°35′47″N, 0°49′23″W) located about 1.5 km away from these mining sites (Supplemental Fig. S1). These tailings are located at a natural park which includes forests of Aleppo pine and endemic xerophytic thickets (Parraga-Aguado et al., 2014). This mining area contains one of the largest Pb and Zn content in the SW of Europe. Average annual rainfall of the area was around 210 mm and 220 mm during 2012 and 2013, respectively (Supplemental Fig. S2), and potential evapotranspiration (ET_o) exceeded rainfall by sixfold (ET_o was 1312 and 1258 mm yr⁻¹ during 2012 and 2013, respectively [Supplemental Fig. S2]). In these years the sampling date corresponding to September 2012 was that one in which the greatest

rainfall occurred and May 2013 followed a rainy month of April (80 mm rainfall) and was wetter than May 2012 (Supplemental Fig. S2).

Rhizosphere soil and plant samples were collected from four mature trees of similar size per site (Agustin, Mercader, Ripolles, Wikon, and non mining-impacted forest) and sampling date. Six to ten small branches, with current and one year-old needles, were cut from different directions from the upper third of the crown of each tree (Supplemental Fig. S3). In the laboratory, the current-year foliage was removed and only the old (one-year) needles were selected (Supplemental Fig. S3). In all sampling periods, at least 200 g of one year-old needles of each population were washed thoroughly with tap and distilled water, gently blotted on filter paper. Then, the mixed samples were randomly divided into five groups. One group was used to determine the needle relative water content (RWC), and each one of the remaining four groups, were divided into two subsamples, one of them was immediately frozen in liquid nitrogen, and stored at -80°C for biochemical analysis, and, the second one was dried at 60°C for 72 h for elemental analysis. At the beginning of the sampling period (i.e., May 2012), four soil subsamples at each site were collected from the rhizosphere of Aleppo pine trees, taking the top 20 cm of soil, and transferred under aseptic conditions to the laboratory.

2.2. Rhizosphere soil analysis

The collected rhizosphere soil samples ($n = 4$ per site) were air-dried, sieved through a 2 mm-sieve mesh, and subjected to several physicochemical analyses including pH, electrical conductivity (EC), equivalent calcium carbonate (% CaCO_3), organic carbon (OC), dissolved organic carbon (DOC), total nitrogen (TN), soil texture, water soluble ions (Cl^- , SO_4^{2-} , Na^+ , K^+ , Ca^{2+} , and Mg^{2+}), and metal(loid) concentrations (As, Cd, Cu, Mn, Ni, Pb, Zn, and Sb) (Parraga-Aguado et al., 2014). (See “Supplementary Material” for a full experimental procedure).

2.3. Elemental analysis in needle samples

About 500 mg of leaf-dried biomass ($n = 4$ per site) were milled to powder in an analytical mill (IKA A11 basic). Samples were incinerated (550°C for 3 h) prior to adding 1 mL of concentrated HNO_3 (65%, Merck, Suprapur). Digestion of the blank sample and reference materials (CTA-VTL-2 certified material, Virginia tobacco leaves) were carried out in the same way. Metal(loid) concentrations (As, Cd, Cu, Mn, Ni, Pb, Zn and Sb) were determined by inductively coupled plasma-mass spectrometry (ICP-MS, Agilent 7500A) (Parraga-Aguado et al., 2014). Chloride, phosphate and sulfate were assessed by ion chromatography (Metrohm). Calcium, magnesium, potassium and sodium were determined by flame atomic absorption spectrometry (FAAS) using a Unicam 969 AA spectrometer. Nitrogen contents were measured on a PDZ Europa ANCA-GSL elemental analyzer (Sercon Ltd., Cheshire, UK).

2.4. Physiological status measurements

All the spectrophotometric determinations were done in quadruplicate using a 96-well plate reader (Multiskan GO, Thermo Scientific). Calibration curves were generated for each assay session using the corresponding standard solutions. A good linearity ($r^2 > 0.99$) between standard concentration and absorbance was observed for all the methods assayed. Four replicate samples were taken per each site and sampling date all the biochemical/physiological determinations were repeated three times. (See “Supplementary material” for a full experimental procedure).

The evaluation of the physiological status of the Aleppo pine populations was carried out by measuring needle relative water content (RWC), photosynthetic pigment concentrations, total soluble protein levels, soluble sugars and starch contents as previously described (López-Orenes et al., 2017). Chlorophyll *a* (Chla), chlorophyll *b* (Chlb),

and total carotenoids were extracted with 100% methanol and quantified as described earlier (Lichtenthaler and Wellburn, 1983).

2.5. Total antioxidant activity determinations

The total antioxidant activity was assessed by three different methods, namely DPPH (2,2-diphenyl-1-picrylhydrazyl radical), $\text{ABTS}^{\bullet+}$ [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonate)], and FRAP (ferric reducing/antioxidant power) assays as previously described (Pérez-Tortosa et al., 2012). DPPH and ABTS radical scavenging activities were expressed as μmol of gallic acid equivalents (GAE) per gram fresh weight. FRAP antioxidant activity was expressed as μmol Fe(II) per gram fresh weight.

2.6. Determination of ascorbate, dehydroascorbate, glutathione, proline and total soluble non-protein thiols

The contents of ascorbate (AA) and dehydroascorbate (DHA) were determined using the α - α' -bipyridyl-based spectrophotometric assay (Gillespie and Ainsworth, 2007). Reduced glutathione (GSH) levels were determined fluorimetrically using an *o*-phthalaldehyde probe (Senft et al., 2000). The concentration of proline (Pro) was determined by the acid-ninhydrin method (Bates et al., 1973), and the levels of total soluble non-protein thiols (NPT) were estimated using the Ellman's reagent (Metwally et al., 2003).

2.7. Determination of hydrogen peroxide, superoxide radicals, lipid peroxidation and protein oxidation

The determination of hydrogen peroxide was carried out by the ferrous ion oxidation–xylenol orange (FOX) method (Cheeseman, 2006). Superoxide anion radical concentrations were measured by the conversion of hydroxylamine into nitrite, and quantified spectrophotometrically at 540 nm after azo coupling with sulfanilamide and naphthylamine (Jiang and Zhang, 2001). Lipid peroxidation products were determined by measuring the concentration of malondialdehyde (MDA), as the end product of the lipid peroxidation process, using the thiobarbituric acid reactive method (Hodges et al., 1999). Protein oxidation was quantified by measuring the protein carbonyl content using the dinitrophenylhydrazine assay (Levine et al., 1994).

2.8. Quantification of total soluble phenolic compounds, total flavonoids, flavanols, hydroxycinnamic acids, lignin, and cell wall-associated proanthocyanidins

The concentration of total soluble phenolic compounds (TPC) was determined in needle methanolic extracts by the Folin-Ciocalteu method (Everette et al., 2010) using gallic acid as standard. The determination of total soluble flavonoids (FO) was carried out according to the aluminum chloride assay using rutin as standard (Kim et al., 2003). Total flavanol content (FA) was assessed using the *p*-dimethylaminocinnamaldehyde (DMACA) reagent and (+)-catechin as a reference (López-Arnaldos et al., 2001). Total hydroxycinnamic acids (HCAs) were measured using the Arnow's reagent and caffeic acid as standard. The pellets of the methanol extracts were used for lignin determination using thioglycolic acid (TGA) using a standard curve with alkali lignin (López-Orenes et al., 2013). The content of cell wall-associated proanthocyanidins (PAs) was determined as described by Vermerris and Nicholson (2006).

2.9. Enzymatic assays

The extraction and assay of PAL (EC 4.3.1.24) and soluble and ionically-bound cell wall Class III peroxidase activities (EC 1.11.1.7; hydrogen donor: H_2O_2 oxidoreductase, PRXs) in needle samples were performed as previously described (López-Orenes et al., 2013). Protein

concentration was determined by using the Bradford protein assay kit (Bio-Rad Laboratories) and bovine serum albumin as standard.

2.10. Quantification of free and conjugated salicylic acid

Quantification of free salicylic acid (SA) and conjugated SA (SAG, 2-O- β -D-glucosylsalicylic acid) were performed using the SA biosensor strain *Acinetobacter* sp. ADPWlux (Huang et al. 2006 and 2005) with some modifications (see “Supplementary material” for a full experimental procedure).

2.11. Statistical analysis

Exploratory analysis of experimental data have been carried out by box-and-whisker plots to compare populations and to detect outliers. Normal probability plots were also made to analyze the normality of data. Soil data were subjected to a one-way ANOVA with site (Agustin, Mercader, Ripolles, Wikon and non mining-impacted forest) as factor, and when the differences were significant at $P \leq 0.05$, a Tukey's HSD post-hoc test was conducted to detect differences between means. The resulting P-values were adjusted using the Benjamini and Hochberg method for multiple comparisons. For the multivariate analysis, log-transformed data were scaled and mean-centered to avoid the effect of the scale on the measurements of the data Dimensionality reduction and classification methods such as principal component analysis (PCA), partial least squares-discriminant analysis (PLS-DA), or random forest (RF) were applied to all data sets. Finally, a heatmap analysis, combined with an agglomerative hierarchical clustering, using the complete-linkage and a distance based on Spearman rank correlation coefficient, were performed to detect differences of the parameters measured in pine needles between mining and NM populations. The clustering methods were visualized by using dendrogram-graphs where the grouped data in the same branch represent similar data. All statistical analysis and graphs were performed using the R Statistical Programming Environment (<https://www.R-project.org/>). (See “Supplementary material”).

3. Results

3.1. Multivariate analysis of rhizosphere soil data

The results of soil analysis for each Aleppo pine population are given in Table 1. Soil pH remained around neutral to slightly alkaline in all collected samples. Mining rhizosphere soils were characterized by sandy texture, lower TN and DOC contents, higher EC values [equivalent to 5–20 dS m⁻¹ in saturated paste (Walker and Bernal, 2008)], and elevated contents of water extractable divalent ions (SO₄²⁻, Ca²⁺, and Mg²⁺) and metal(loid)s (particularly As, Pb and Zn). Significant negative correlations were found between soil fertility parameters (TN and DOC) and both divalent ion concentrations and sand percentages ($-0.95 > r > -0.6$, $P < 0.0001$; Supplemental Table S1). Moreover, water extractable SO₄²⁻ and Ca²⁺ levels exhibited a strong correlation ($r = 0.97$, $P < 0.0001$; Supplemental Table S1), which can be linked to the secondary formation of gypsum in the tailings as previously outlined (Parraga-Aguado et al., 2014). In general, monovalent ions (Cl⁻, K⁺, and Na⁺) remained closer to the values found in control soils, except for Na in Agustin tailings. Rhizosphere mining samples showed 15- to 40-fold higher levels of total metal(loid) concentrations than non-mining (NM) samples. Within mining soils, Ripolles and Wikon exhibited the highest levels of Cu, Pb and Zn, and the lowest levels of K⁺ and Mg²⁺ when compared with Agustin and Mercader rhizosphere soils. Mercader soil showed relatively low metal concentrations and the highest TN and organic carbon contents (Table 1).

Results of PCA and PLS-DA revealed differences between non-mining and mining soils due to the first component (Supplemental Fig. S4). The plot also showed a clear clustering between Mercader and Agustin samples and, Ripolles and Wikon samples. These differences indicated close soil physicochemical similarities between samples sorted into the same cluster (Supplemental Fig. S4). The first component of both PCA and PLS-DA, which explained ~62% of the total variance of the data set, was mainly influenced by soil fertility parameters (DOC and TN) and by metal(loid) soil concentrations (mainly Pb, Zn, As, and Cu). The second component of both PCA and PLS-DA (~10% of

Table 1
Physicochemical characteristics and metal(loid) concentrations in soils from both mining (Agustin, Mercader, Ripolles and Wikon) and non-mining (NM) samples. Data represent means (n = 4) \pm SE. Values followed by different letters in a row are significantly different at $P < 0.05$, Tukey's HSD post-hoc test.

	Rhizosphere soil sample				
	NM	Agustin	Mercader	Ripolles	Wikon
Soil parameters					
pH (1:5)	7.99 \pm 0.05 ^a	7.50 \pm 0.06 ^b	7.53 \pm 0.18 ^b	7.44 \pm 0.06 ^{bc}	7.12 \pm 0.03 ^c
EC (1:5) (dS m ⁻¹)	0.27 \pm 0.02 ^c	1.31 \pm 0.45 ^{bc}	1.44 \pm 0.53 ^b	0.80 \pm 0.15 ^{bc}	2.81 \pm 0.05 ^a
CaCO ₃ (%)	20.32 \pm 0.64 ^a	12.14 \pm 3.58 ^b	9.23 \pm 0.34 ^b	10.10 \pm 1.76 ^b	6.27 \pm 0.09 ^b
OC (%)	0.78 \pm 0.09 ^{ab}	0.69 \pm 0.02 ^{ab}	0.99 \pm 0.13 ^a	0.59 \pm 0.09 ^b	0.50 \pm 0.05 ^b
DOC (mg kg ⁻¹)	108.96 \pm 18.24 ^a	52.61 \pm 4.65 ^b	70.15 \pm 7.51 ^{ab}	63.11 \pm 12.61 ^{ab}	44.97 \pm 12.01 ^b
TN (g kg ⁻¹)	0.91 \pm 0.07 ^a	0.32 \pm 0.05 ^{bc}	0.59 \pm 0.15 ^b	0.32 \pm 0.06 ^{bc}	0.26 \pm 0.04 ^c
Texture (%)					
Clay	27.51 \pm 1.16 ^a	1.08 \pm 0.00 ^c	2.10 \pm 0.48 ^b	1.00 \pm 0.00 ^c	3.72 \pm 0.68 ^b
Silt	37.10 \pm 4.31 ^a	13.97 \pm 1.53 ^c	19.29 \pm 2.53 ^b	10.77 \pm 2.17 ^c	14.56 \pm 1.51 ^{bc}
Sand	35.39 \pm 3.20 ^c	84.95 \pm 1.53 ^a	78.62 \pm 2.64 ^b	88.23 \pm 2.17 ^a	81.72 \pm 1.43 ^b
Water extractable ions (1:5) (mg L⁻¹)					
Cl ⁻	19.82 \pm 2.91 ^a	11.94 \pm 0.76 ^{ab}	8.72 \pm 0.66 ^b	9.39 \pm 0.76 ^b	14.80 \pm 2.60 ^{ab}
SO ₄ ²⁻	20.83 \pm 0.58 ^c	1405.00 \pm 134.46 ^a	676.08 \pm 318.34 ^b	295.91 \pm 75.97 ^{bc}	1610.99 \pm 16.84 ^a
Na ⁺	17.20 \pm 2.76 ^b	94.72 \pm 14.87 ^a	17.35 \pm 0.90 ^b	11.42 \pm 0.72 ^b	18.46 \pm 6.63 ^b
K ⁺	12.70 \pm 1.24 ^{ab}	14.95 \pm 2.99 ^a	13.09 \pm 0.33 ^a	7.22 \pm 0.65 ^{bc}	6.95 \pm 0.49 ^c
Ca ²⁺	35.15 \pm 0.25 ^b	508.71 \pm 53.44 ^a	240.74 \pm 104.67 ^b	118.72 \pm 37.95 ^b	630.18 \pm 25.86 ^a
Mg ²⁺	2.67 \pm 0.44 ^b	27.70 \pm 6.45 ^a	35.13 \pm 9.77 ^a	17.14 \pm 6.95 ^{ab}	17.08 \pm 2.23 ^{ab}
Total metal(loid)s (mg kg⁻¹)					
As	18.25 \pm 11.38 ^d	226.67 \pm 23.63 ^c	697.67 \pm 31.39 ^{ab}	559.75 \pm 78.31 ^b	800.50 \pm 46.19 ^a
Cd	17.25 \pm 11.25 ^a	36.00 \pm 10.21 ^a	22.67 \pm 7.22 ^a	33.75 \pm 4.85 ^a	45.50 \pm 7.14 ^a
Cu	44.83 \pm 2.83 ^b	86.47 \pm 2.41 ^a	95.00 \pm 0.87 ^a	114.38 \pm 14.82 ^a	104.50 \pm 3.66 ^a
Mn	1635.00 \pm 61.71 ^c	7639.00 \pm 442.22 ^b	10,058.33 \pm 96.25 ^a	9628.75 \pm 570.83 ^a	10,198.00 \pm 275.29 ^a
Ni	39.25 \pm 1.18 ^a	33.67 \pm 2.26 ^{ab}	27.67 \pm 1.89 ^{bc}	28.25 \pm 2.29 ^{bc}	24.25 \pm 2.66 ^c
Pb	397.50 \pm 23.39 ^b	4665.33 \pm 307.89 ^a	2873.33 \pm 208.83 ^a	6288.25 \pm 1779.73 ^a	5713.50 \pm 329.77 ^a
Zn	264.25 \pm 16.07 ^c	8685.33 \pm 1095.77 ^a	4356.00 \pm 245.83 ^b	9376.00 \pm 381.55 ^a	9505.00 \pm 440.54 ^a
Sb	1.00 \pm 0.00 ^c	49.00 \pm 12.26 ^b	60.67 \pm 10.50 ^{ab}	66.50 \pm 8.58 ^{ab}	98.50 \pm 11.55 ^a

total variance) was influenced by K and Na levels and by organic carbon content (OC) (Supplemental Fig. S4).

3.2. Needle ionome of Aleppo pine populations

PCA on needle ionome data revealed two principal components (PC) that together explained ~57% of the total variance (Fig. 1). Similar results were obtained when the data were analyzed using PLS-DA (Supplemental Fig. S5). The first PC, which explained ~41% of the total variance, clearly separated NM from mining samples, and was mainly influenced by foliar Mg and S and by metal(loid) concentrations (Pb, Zn, As, and Cd). The scatterplot of the first two principal components provided a clear separation between mining samples in the different seasons, and the highest positive influence on the PC2 was given by monovalent ions. The main differences between seasons were associated with N, S and Ca in spring and Cl^- , Na^+ and K^+ in summer.

Foliar concentration of macronutrient contents in both NM and mining samples were below the normal average values reported for *P. halepensis* grown on non-polluted soils (Clarke et al., 2008) (Supplemental Table S2), indicating a low nutritional status of pine trees. Foliar concentrations of As, Mn, Ni, Pb, and Zn in mining needle samples were between 3 and 10-fold higher than those found in NM samples (Supplemental Tables S2 and S3). However, the accumulated metal(loid) levels were within the reported normal range for *Pinus* species (Clarke et al., 2008; Pratas et al., 2005), with the notable exception of Pb and Zn, which exceeded the critical threshold value of 5.59 and 77.55 $\mu\text{g g}^{-1}$, respectively, especially in spring needle samples (Supplemental Table S2).

3.3. Multivariate analysis of physiological and antioxidative/oxidative data in Aleppo pine needles

Physiological and antioxidative/oxidative data were subjected to multivariate statistical analysis. Firstly, a PCA was carried out, where the first two components explained ~50% of the total variance (Fig. 2). This plot showed a clear separation between spring and summer samples. The PCA also revealed evident clustering differences between NM samples, that were situated in the lower left-quadrant, and mining samples that were positioned in both the upper left- and the lower right-quadrant (Fig. 2). The main differences between NM and mining populations were associated with soluble phenolic compounds [flavanols (FA), flavonoids (FO), HCAs and total phenol content (TPC)], total antioxidant activities (FRAP, ABTS and DPPH), ascorbate (AA) and protein

content. The main differences between seasons were associated with GSH, chlorophyll and sugar levels, proanthocyanidins (PAs) and soluble peroxidase activity (sPRX) in spring and carotenoids and needle relative water content (RWC) in summer.

Physiological and biochemical data were further processed by PLS-DA method. PLS-DA results were similar to those obtained using PCA (see Supplemental Fig. S6). PLS-DA provides a quantitative estimation of the most influential parameters based on the variable importance in the projection (VIP). Variables with VIP scores >1 were identified as the most important markers according to their ability to discriminate among groups. In order to visualize at a glance the most significant markers, the correlation coefficients for the first two components of PLS-DA and VIP score were plotted. As seen in Fig. 3A, markers with the VIP threshold >1 and with high correlation with PLS-DA1, accounting for ~32% of the variation, were those related with plant growth performance (RWC, chlorophyll and sugar contents), antioxidant compounds (AA, GSH, FA, and PAs), DPPH radical scavenging activities, and soluble peroxidase activity (sPRX). Parameters highly correlated with PLS-DA2, accounting for ~18% of the variation, and with the VIP threshold >1 were malondialdehyde (MDA), used as a lipid oxidation marker, and again chlorophylls, proteins, antioxidant compounds (AA, HCAs), DPPH radical scavenging activities, and PRX activities.

Then, random forest (RF) algorithm was also used to identify important variables based on mean decrease in accuracy criterion. As shown in Fig. 3B the variables with the greatest effect (mean decrease in accuracy ≥ 20) were PAs, GSH, photosynthetic pigments, proline and RWC, followed by a set of 11 variables (mean decrease in accuracy ≥ 15) related with phenol metabolism [PRX activities, lignin, HCAs, flavanols (FA)], AA and ROS levels (H_2O_2 , $\text{O}_2^{\cdot-}$), and lipid oxidation (MDA).

To determine in what extent seasonal changes affected the antioxidative/oxidative profile in the different Aleppo pine populations studied, the ratio values (mining/non-mining) were log2-transformed and a two-way complete-linkage hierarchical clustering was performed by using a distance defined in terms of Spearman rank correlation and represented in a heatmap (Fig. 4). The statistical significance of fold changes was determined by a non-parametric Wilcoxon's test, and the mean values obtained are shown in Supplemental Table S5. The dendrogram showed a clear separation between spring and summer samples, which was already visible in the PCA analysis (see, Fig. 2), as well as a marked separation of Mercader samples from those of other populations, especially in spring. Strong separation of the Mercader samples was caused by an increase in chlorophylls, and GSH and by the absence

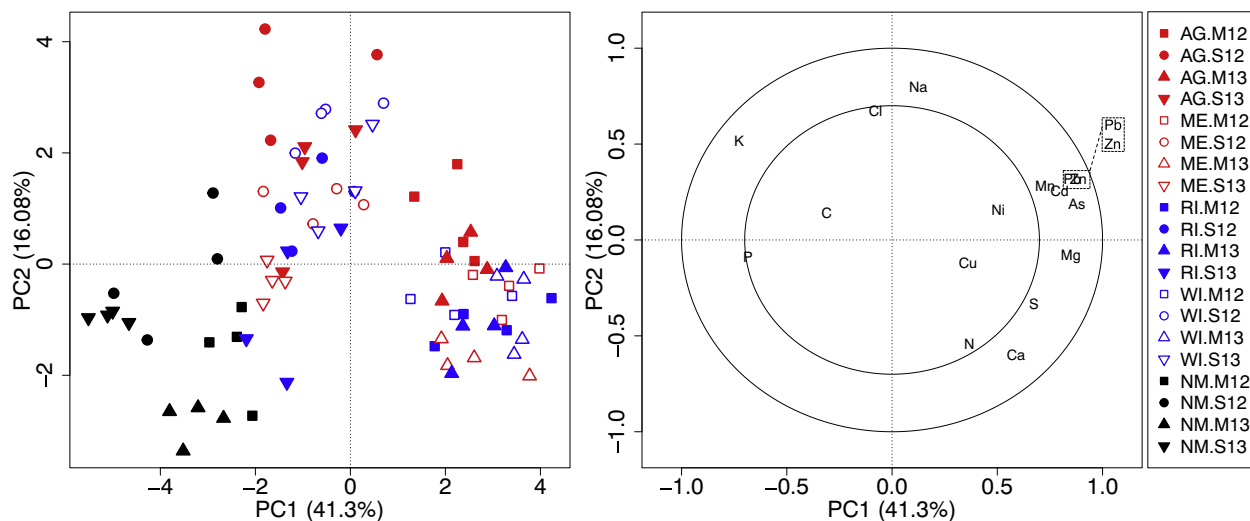


Fig. 1. Principal component analysis based on correlation matrix applied to needle ionome data of non-mining (NM) and mining Aleppo pine populations, in late spring and summer in 2012 and 2013. Circles represent $r^2 = 50\%$ and 100% variability explained by the components. Population codes: NM, filled black color; Agustín, filled red color; Mercader, unfilled red color; Ripolles, filled blue color; and Wikon, unfilled blue color. Season and year codes: May 2012, squares; September 2012, circles; May 2013, triangles; September 2013, inverted triangles.

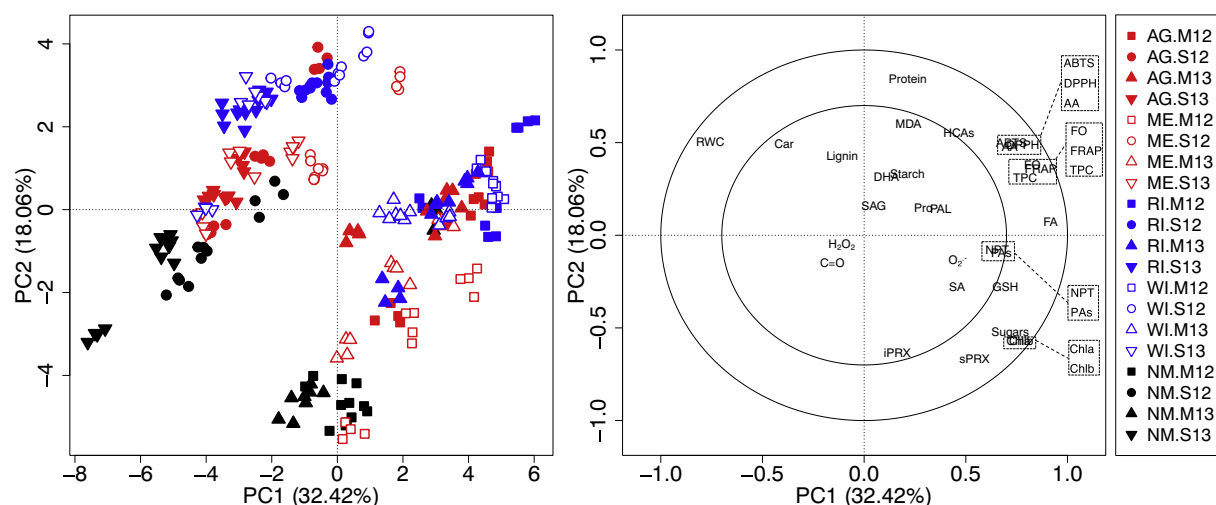


Fig. 2. Score (left) and correlation (right) plots of the first two components of the PCA applied to physiological and biochemical variables measured in needles of Aleppo pine trees growing in non-mining (NM) and mining tailings pile (Agustin, Mercader, Ripolles and Wikon) in late spring and summer in 2012 and 2013. Circles represent $r^2 = 50\%$ and 100% variability explained by the components. For population, season and year codes, see legend to Fig. 1. Abbreviations: AA, ascorbate; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation scavenging activity; Car, total carotenoids; Chla, Chlorophyll *a*; Chlb, chlorophyll *b*; C=O, protein carbonyl group content; DHA, dehydroascorbate; DPPH, 1,1-Diphenyl-2-picrylhydrazyl radical scavenging activity; FA, total flavanols; FO, total flavonoids; FRAP, ferric reducing/antioxidant power; iPRX, ionically-bound cell wall class III plant peroxidase activity; H_2O_2 , hydrogen peroxide; HCAs, hydroxycinnamic acids; MDA, malondialdehyde; NPT, total soluble non-protein thiols; $O_2^{\cdot-}$, superoxide radical; PAL, phenylalanine ammonia-lyase activity; PAs, cell wall-associated proanthocyanidins; Pro, proline; RWC, relative water content; SA, salicylic acid; SAG, 2-O- β -D-glucosylsalicylic acid; sPRX, soluble class III plant peroxidase activity; TPC, total phenol content.

of change in total antioxidant activities, soluble phenols (TPC and FO), and iPRX activities (see Supplemental Table S4). Besides, there was a marked seasonal difference in total carotenoids/chlorophylls ratio among mining samples, particularly in Ripolles and Wikon samples (Supplemental Fig. S7). The dendrogram also revealed that Agustin summer samples were separated from those of Wikon and Ripolles. This separation can be caused by the reduction in the levels of photosynthetic pigments, although in spring these populations did not diverge and were clustered according to the year of sampling (Fig. 4).

Biochemical markers, illustrated in the rows of the heatmap in Fig. 4, were clustered into two large groups. The first group was characterized by variables associated with the physiological status (photosynthetic pigments, RWC, sugars and starch contents; group 1, Fig. 4). The second group was characterized by markers related with phenol metabolism (HCAs, TPC, FA, FO, PRX and PAL activities), total antioxidant activities (FRAP, ABTS and DPPH), and AA content (group 2, Fig. 4). The heatmap also showed that the amplitude of changes in the biochemical markers clustered in the group 2 was larger than that of the group 1 during spring and summer seasons. These results indicated the important role of antioxidants and phenol metabolism in mining Aleppo pine populations (Fig. 4 and Supplemental Table S4). In fact, although a marked increase in the levels of DHA was noted particularly in spring (Fig. 4), all mining needles exhibited a high AA redox ratio $[AA/(AA+DHA)]$ (Supplemental Fig. S6). This ratio is reported to be an important indicator of the redox status of the plant cell (Foyer and Noctor, 2016).

Finally, no general changes were observed in the endogenous levels of the stress-related phytohormone salicylic acid (SA) between NM and mining populations (Fig. 4 and Supplemental Table S4), although a slight increase in SA levels was found in Mercader and Agustin summer samples. In general, conjugated SA (SAG) tended to decrease during the wetter spring period (i.e., May 2013) and to increase during the wetter summer period (i.e., September 2012).

4. Discussion

The present study aimed at evaluating the metabolic adjustments in response to the harsh conditions prevailing in mine-tailings under semi-arid Mediterranean conditions in four different mining Aleppo pine populations. Here, PCA and PLS-DA for rhizosphere soil data

showed a clear separation between NM and mining soils. Within mining soils, the main differences were related with the levels of K^+ , Na^+ , Pb and Zn (Table 1 and Fig. S4). Moreover, PCA performed on needle ionome data discriminates between NM and mining populations, although cannot discriminate within mining pine populations (Fig. 1). Several studies have described that foliar ionome can be used as a rapid tool for biomonitoring HMMs contamination in highly polluted areas (Madejón et al., 2006, and refs. herein). Here, there were no good correlations between HMM concentrations in mining soils and their corresponding concentrations in pine needles (Fig. 1 and Table S1). These data are in agreement with those reported by Parraga-Aguado et al. (2014) for the same tree species within the same mining area. A plausible explanation for the lack of correlation between HMM concentrations in soils and needles could be related to the fact that Mediterranean Aleppo pine is well adapted to withstand drought because of its dimorphic root system (Voltas et al., 2015). In fact, stable isotopes studies have supplied evidence of the ability of this species to acquire mineral elements at different soil depth when surface soil layers become dry (Voltas et al., 2015). Further investigation is needed to verify this assumption.

In our study, foliar concentrations of some nutrients (N, S, Mg, Mn and Ca) and metal(loid)s were higher in spring than in summer (Fig. 1). The accumulation of these nutrients can be related to the spring growth period of this species in the Mediterranean area (Pacheco et al., 2017). An increase in the accumulation of K^+ was observed in summer needles. Potassium is the most abundant cation in plant cells, and plays a key role in the water economy of plants, particularly by the maintenance of cell turgor (Marschner, 1995; Shabala and Pottosin, 2014). Our results revealed a strong correlation ($r > 0.8$, $P < 0.0001$, Supplemental Table S1) between K levels and needle RWC. Besides K^+ , both foliar Na^+ and Cl^- levels also exhibited correlations with RWC higher than 0.4 ($P < 0.0001$) (Supplemental Table S1). These results suggested that the uptake of Na^+ together with the counterion Cl^- could also contribute to osmotic adjustment during summer in mining samples. Aleppo pine is a moderately salt-tolerant tree (Parraga-Aguado et al., 2014). There is extensive evidence that salt-tolerant species could partially substitute K^+ for Na^+ , particularly under low K soils (Battie-Laclau et al., 2014; Erel et al., 2014; Marschner, 1995), which are in line with our findings. Despite the fact that soluble sugars and proline

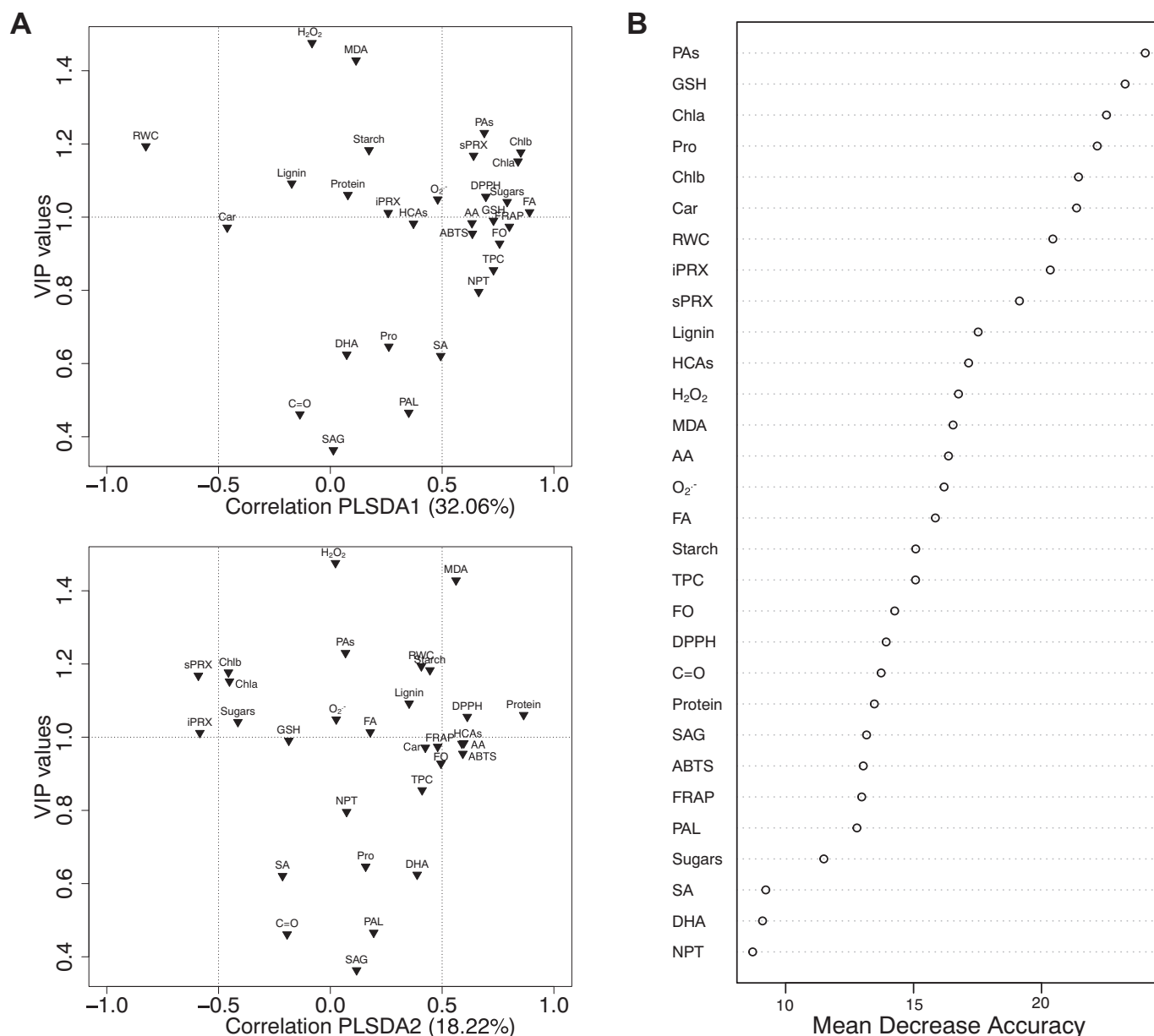


Fig. 3. Identification of the most influential physiological and biochemical biomarkers based on the variable importance in the projection (VIP) and the correlation coefficients for the first three components of PLS-DA (A), and on mean decrease in accuracy estimated by random forest machine learning algorithm (B). For abbreviations, see legend to Fig. 2.

are considered key osmolytes contributing to osmotic adjustment in stressed plants (Suzuki et al., 2014), negative or non-significant correlations between RWC and soluble sugars or proline were found (Supplemental Table S1). These results suggest that these organic solutes appear to play a minor role in the needle osmotic adjustment in Aleppo pine populations studied. Osmotic adjustment via the accumulation of inorganic ions requires a lower energetic cost. Therefore, the accumulation of inorganic ions can be considered an efficient carbon-saving strategy for maintaining cell turgor during the dry Mediterranean summers under conditions of reduced photosynthetic potential. The advantages to using inorganic ions for cell osmotic adjustment under different stress conditions have been previously reported in different plant species (Orsini et al., 2011; Shabala and Lew, 2002).

In general, the foliar concentrations of K, N, and P found in pine populations were below the normal range reported for this species (Clarke et al., 2008) and lower than those described for several populations of Aleppo pine grown under similar semi-arid Mediterranean conditions

(López-Serrano et al., 2005; Querejeta et al., 2008). These results suggest that K, N, and P are likely to be growth-limiting factors in this species and can be related to the nutrient-limited conditions of the tailings. In contrast, the foliar amount of S accumulated in mining pine populations exceeded by more than twice the normal range (Clarke et al., 2008) (Tables S2 and S3). S content was correlated with the levels of S-rich molecules (i.e., the non-protein thiol pool, $r > 0.4$, $P < 0.0001$, and GSH content, $r > 0.6$, $P < 0.0001$, Supplemental Table S1) found in needles.

Actually, the examination of overall antioxidative/oxidative profile evidenced that spring needles, which showed higher accumulation of metal(loid)s, were characterized by high GSH levels. Interestingly, GSH was ranked as one of the most important variables based on VIP score and mean decrease in accuracy criterion in RF analysis (Fig. 3). Moreover, foliar HMM concentrations exhibited correlations with GSH higher than 0.4 ($P < 0.0001$) (Supplemental Table S1). High GSH content is considered essential for detoxification of HMMs (Hernández et al.,

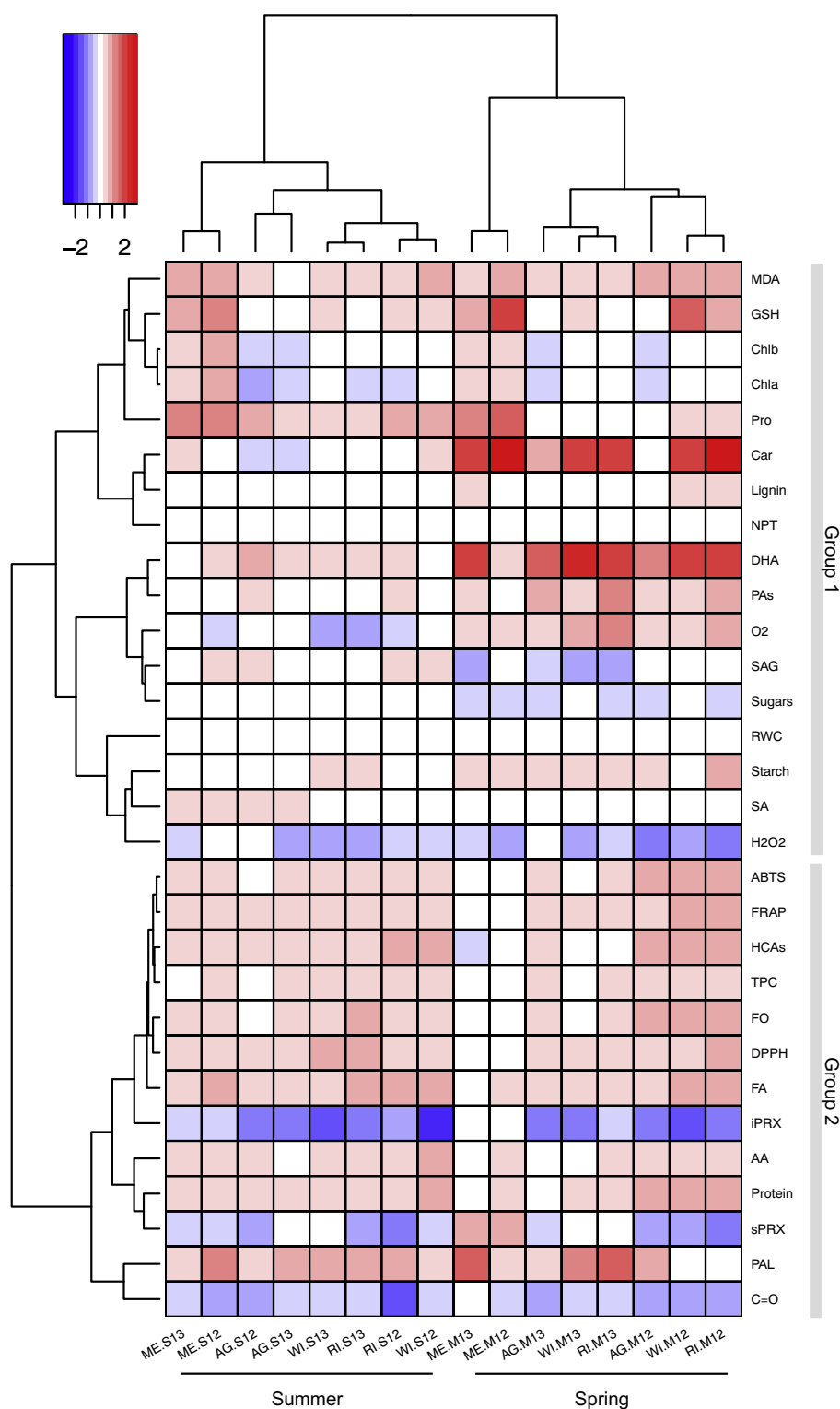


Fig. 4. Heatmap and complete-linkage hierarchical clustering (by using a distance based on Spearman rank correlation coefficient) showing the seasonal fold change (mining vs. non-mining) of the physiological and biochemical parameters measured in needles of Aleppo pine trees growing in non-mining and in mining tailings piles in late spring and summer in 2012 and 2013. Log2 ratios of fold changes relative to each respective control group are given by shades of red or blue colors according to the scale bar. For abbreviations, see legend to Fig. 2.

2015; Jozefczak et al., 2012). Thus, taken together, these results suggest that thiol-mediated complexation could be an important mechanism of metal(loid) detoxification in Aleppo pines under these edaphoclimatic conditions.

Proline was also placed among the top-5 significant biomarkers in RF analysis (Fig. 3B). Extensive evidence suggests that proline is a potent

non-enzymatic antioxidant and contributes to the stabilization of redox systems such as the GSH pool (Liang et al., 2013; Szabados and Saviouré, 2010). Our results showed a good correlation between proline and GSH levels ($r > 0.58$, $P < 0.0001$, Supplemental Table S1), which is in line with previous results demonstrating that the up-regulation of proline increased the levels of reduced GSH (for review see, Liang et al.,

2013), thus reinforcing the above proposed essential role of GSH in HMM detoxification mechanisms in Aleppo pine.

Spring mining needles were also typified by increased concentrations of total soluble phenols (TPC), HCAs, flavonoids, flavanols, and particularly flavan-3-ol polymers (PAs) (Figs. 1 and 4). It is well established that nutrient deficiency and HMM stress lead to the accumulation of phenolic compounds by altering phenol metabolizing enzymes such as PAL and PRX (Boudet, 2007; Dixon and Paiva, 1995; Gill and Tuteja, 2010; Jouili et al., 2011). The data presented confirm the induction of PAL activity in all mining samples (Fig. 4 and Table S4). In its turn, soluble PRX (sPRX) and cell wall-bound PRX (iPRX) activities showed a different pattern in Mercader as compared with the other mining samples (Fig. 4 and Table S4). In particular, sPRX activity increased in Mercader but decreased or unchanged in the other mining samples, whereas iPRX activity remained unaffected in Mercader but decreased in the other populations. Taken together, these results suggest that the lower levels of soluble phenolics (HCAs, flavonoids, and TPC) accumulated in Mercader needles could be related with the different behavior of PRX and PAL activities found in this populations. Moreover, it is worth noting that the contents of secondary metabolites are affected by foliar N levels (Fritz et al., 2006). Therefore, the lower levels of soluble foliar phenolics found in Mercader samples can also be related with their higher foliar N concentrations.

Phenolics have been described to be efficient antioxidants (Rice-Evans et al., 1997). Phenol compounds are able to chelate metals, quench lipid peroxidation, scavenge ROS and to protect or recycle endogenous antioxidants, such α -tocopherol and ascorbate (Agati et al., 2012; Michalak, 2006). These antioxidant properties of phenolics depend on their structure, mostly on the number and position of hydroxyl groups bound to the aromatic ring (Andejejkovic et al., 2006; Rice-Evans et al., 1997). In this way, the presence of *o*-diphenol groups enhances the radical scavenging and metal-chelating capacities of phenolic compounds. Increased accumulation of phenolics has previously been described in *Pinus* species challenged with heavy metals (Kareolewski and Giertych, 1995; Roitto et al., 2005). In our study, high correlations between phenolic compounds and foliar accumulation of metal(loid)s were noticed. These results suggest that phenolics could have a role in detoxification and accumulation of metal(loid)s by functioning as chelating ligands. Noteworthy, the oligomeric phenolics PAs were ranked as the most important biomarker in RF analysis (Fig. 3B). This fact, together with the trend observed in seasonal accumulation in needles, with higher accumulation in spring (Fig. 4), which coincided with higher foliar accumulation of HMMs (Table S3), seems to assign a relevant role to PAs in Aleppo pine tolerance to harsh mine tailings conditions.

Apart from GSH and phenolics, mining needles also showed higher antioxidant capacity, estimated by ABTS, DPPH and FRAP tests. Higher AA levels and a high AA redox ratio were also observed in these samples (Figs. 4 and S8). The increase in the levels of AA, GSH, proline, and phenolics seemed to be effective in controlling $O_2^{\bullet-}$ and H_2O_2 levels in both seasons. In fact, a reduction in protein oxidation, measured as protein carbonyl content, was observed. Nevertheless our data also revealed a certain degree of lipid peroxidation in foliar mining samples in both seasons, as compared with NM ones. This lipid peroxidation appeared not to have a significant detrimental effect on photosynthetic metabolism, as evidenced by the higher starch concentrations found in mining samples ($\sim 300 \mu\text{g g}^{-1}$ FW) compared to NM ones ($\sim 250 \mu\text{g g}^{-1}$ FW). In this regard, it is important to highlight both the marked increase in the carotenoid content, particularly in spring, and the decrease in the levels of chlorophylls in summer mining samples. It is well established that carotenoids have a dual role in plants as both accessory light-harvesting pigments and photoprotective molecules by quenching triplet chlorophyll, singlet oxygen (1O_2) and other free radicals (Young, 1991). Chlorophylls in the triplet excited state can lead to the formation of 1O_2 , especially under high irradiance as well as under stress conditions that induce closing of stomata (Gill and Tuteja, 2010). Thus, the increase

observed in the ratio of total carotenoids to chlorophylls, especially in summer and in Ripolles and Wikon samples, can be considered an adaptation strategy to prevent irreversible damage to the photosynthetic apparatus. The increase in the ratio carotenoids/chlorophylls can contribute, on the one hand, to reduce light absorption and 1O_2 formation, and on the other hand, to increase the photoprotection capacity of needles under these drastic concurrent stressful conditions, as previously outlined (Haldimann, 1998). It is worthy to mention that the low levels of chlorophylls found in Agustin summer needles can be related to the particularly low levels of N found in these samples. This view is reinforced by the trend observed in Mercader samples, whose N levels were the highest in all populations and also showed the highest levels of chlorophylls.

Finally, as water scarcity is characteristic in semi-arid Mediterranean regions, it is not surprising to found that rainfalls favored Aleppo pine growth. In fact, several studies have highlighted that long-lived tree species from Mediterranean climates are plastic enough to cope with erratic rainfall patterns (Camarero et al., 2010).

5. Conclusions

Our results revealed a strong seasonality in needle ionome and antioxidative/oxidative profiles that correlates with the seasonal variation of growth rate in Mediterranean Aleppo trees. Mining samples exhibited higher contents of antioxidative metabolites (AA and soluble phenols) than NM ones in the two seasons studied. The associated higher antioxidant activity could contribute to prevent oxidative injury induced by the higher concentrations of HMM found in these populations. Moreover, the magnitude of the changes in the physiological and antioxidative/oxidative parameters was more pronounced in spring samples, coinciding with the period of active growth of Mediterranean Aleppo pine. Despite the fact that spring needles accumulated higher levels of HMMs, particularly Pb and Zn, pine performance seem not to be affected. Data analysis assigned to PAs and GSH a key role in spring needle metabolism. This could be related to the involvement of these compounds in HMM detoxification mechanisms in Aleppo pines. During summer, needles maintained high RWC through the accumulation of inorganic ions and increased photoprotection capacity what seem to be critical for tree acclimation to the dry season.

Predicted precipitation scenarios in the Mediterranean basin within the context of climate change, with significant rainfall reductions, would lead to a shift in the metabolic behavior of Aleppo pines growing on mine tailings. The present study points to future reductions in photosynthetic capacity of pine populations due to the more prolonged dry periods.

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Appendix B. Supplementary data

Supplementary data associated with this article can be found in the online version, at <https://doi.org/10.1016/j.scitotenv.2018.05.049>. These data include the Google map of the most important areas described in this article.

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